

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

I hereby certify that this paper or fee is being deposited on February 11, 1999 with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 as Express Mail Label No. EL154556409US and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231


 William W. Steenburg

MELANOMA ANTIGENIC PEPTIDES

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority under 35 U.S.C. § 119(e) of provisional U.S. Application No. 60/103,229, filed October 5, 1998, the contents of which are hereby incorporated by reference.

TECHNICAL FIELD

This invention relates to the field of human tumor vaccines and in particular, vaccine components useful against human melanoma.

BACKGROUND OF THE INVENTION

Tumor specific T cells, derived from cancer patients, will bind and lyse tumor cells. This specificity is based on their ability to recognize short amino acid sequences (epitopes) presented on the surface of the tumor cells by MHC class I and class II molecules. These epitopes are derived from the proteolytic degradation of intracellular proteins called tumor antigens encoded by genes that are either uniquely or aberrantly expressed in tumor or cancer cells.

The availability of specific anti-tumor T cells has enabled the identification of tumor antigens and subsequently the generation of cancer vaccines designed to provoke an anti-tumor immune response. Anti-tumor T cells are localized within cancer patients, including in the blood (where they can be found in the peripheral blood mononuclear cell fraction), in ascites fluid in ovarian cancer patients (tumor associated lymphocytes or TALs) or within the tumor itself (tumor infiltrating lymphocytes or TILs). Of these, TILs have been the most useful in the identification of tumor antigens and tumor antigen-derived peptides recognized by T cells.

Conventional methods to generate TILs involve mincing tumor biopsy tissue and culturing the cell suspension *in vitro* in the presence of the T cell

growth factor interleukin-2 (IL-2). Over a period of several days, the combination of the tumor cells and IL-2 can stimulate the proliferation of tumor specific T cells at the expense of tumor cells. In this way, the T cell population is expanded. The T cells derived from the first expansion are subsequently mixed with irradiated tumor cells and cultured *in vitro* with IL2 to promote further proliferation and enrichment of tumor reactive T cells. After several rounds of *in vitro* expansion, a potent anti-tumor T cell population can be recovered and used to identify tumor antigens via conventional but tedious expression cloning methodology.

Kawakani, Y. et al. (1994) *PNAS* 91(9):3515-19.

This currently employed methodology used to generate tumor specific T cells *in vitro* is unreliable and the antigens identified by this method do not necessarily induce an anti-tumor immune response. Numerous experiments demonstrate that encounter of antigens by mature T cells often results in the induction of tolerance because of either ignorance, anergy or physical deletion.

Pardoll (1998) *Nature Med.* 4(5):525-531.

Thus, a need exists to identify antigenic peptides that will induce an immune response for the generation of effective anti-cancer vaccines. This invention satisfies this need and provides related advantages as well.

DISCLOSURE OF THE INVENTION

Using a technique known as Solid-PHase Epitope REcovery ("SPHERE") (described in WO 97/35035), synthetic gp 100 melanoma epitopes that are specifically recognized by tumor specific immune effector cells have been identified. Using SPHERE, a library of oligopeptides between 8 to 10 amino acids in length was made and screened for their specificity and ability to raise immune effector cells that specifically target and lyse melanoma cells.

Thus, this invention provides novel, synthetic polypeptide vaccines against human melanoma and methods for making these vaccines. Polynucleotides encoding these polypeptides are further provided herein.

This invention also provides antigen presenting cells (APC) that present the novel polypeptides on the cell surface and use of the APC in cancer therapy. Immune effector cells expanded in the presence of the APC are further provided herein. These compositions are useful as melanoma vaccines and in adoptive immunotherapy.

MODE(S) FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, *e.g.*, Sambrook, et al. *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd edition (1989); *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY* (F. M. Ausubel, et al. eds., (1987)); the series *METHODS IN ENZYMOLOGY* (Academic Press, Inc.): *PCR 2: A PRACTICAL APPROACH* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1989) *ANTIBODIES, A LABORATORY MANUAL*, and *ANIMAL CELL CULTURE* (R.I. Freshney, ed. (1987)).

As used herein, certain terms may have the following defined meanings.

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The terms "polynucleotide" and "nucleic acid molecule" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides, and/or their

analogs. Nucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes, for example, single-, double-stranded and triple helical molecules, a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A nucleic acid molecule may also comprise modified nucleic acid molecules.

"Oligonucleotide" refers to polynucleotides of between about 5 and about 100 nucleotides of single- or double-stranded DNA. Oligonucleotides are also known as oligomers or oligos and may be isolated from genes, or chemically synthesized by methods known in the art.

The term "genetically modified" means containing and/or expressing a foreign gene or nucleic acid sequence which in turn, modifies the genotype or phenotype of the cell or its progeny. In other words, it refers to any addition, deletion or disruption to a cell's endogenous nucleotides.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eukaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook, et al. (1989) *Supra*). Similarly, an eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described below for constructing vectors in general.

A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

A "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors and the like. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, retroviral vector refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form which integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a transgene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. (see, *e.g.*, WO 95/27071) Ads are easy to grow and do not require integration into the host cell genome. Recombinant

Ad-derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. (see, WO 95/00655; WO 95/11984). Wild-type AAV has high infectivity and specificity integrating into the host cells genome. (Hermonat and Muzyczka (1984) *PNAS USA* 81:6466-6470; Lebkowski, et al. (1988) *Mol. Cell. Biol.* 8:3988-3996).

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

Gene delivery vehicles also include several non-viral vectors, including DNA/liposome complexes, and targeted viral protein DNA complexes.

Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, e.g., TCR, CD3 or CD4.

"Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogsteen binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more

extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

As used herein, the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or proliferation. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1 α), interleukin-11 (IL-11), MIP-1 α , leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO) and flt3 ligand. The present invention also includes culture conditions in which one or more cytokine is specifically excluded from the medium. Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

"Co-stimulatory molecules" are involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. One exemplary receptor-ligand pair is the B7 co-stimulatory molecules on the surface of DCs and its counter-receptor CD28 or CTLA-4 on T cells (Freeman, et al. (1993) *Science* 262:909-911; Young, et al. (1992) *J. Clin. Invest.* 90: 229; Nabavi, et al. *Nature* 360:266). Other important co-stimulatory molecules are CD40, CD54, CD80, CD86.

The terms "antigen-presenting cells" or "APCs" includes both intact, whole cells as well as other molecules which are capable of inducing the presentation of one or more antigens, preferably in association with class I MHC molecules. Examples of suitable APCs are discussed in detail below and include, but are not limited to, whole cells such as macrophages, dendritic cells, B cells,

purified MHC class I molecules complexed to β 2-microglobulin; and foster antigen presenting cells.

Dendritic cells (DCs) are potent antigen-presenting cells. It has been shown that DCs provide all the signals required for T cell activation and proliferation. These signals can be categorized into two types. The first type, which gives specificity to the immune response, is mediated through interaction between the T-cell receptor/CD3 ("TCR/CD3") complex and an antigenic peptide presented by a major histocompatibility complex ("MHC") class I or II protein on the surface of APCs. This interaction is necessary, but not sufficient, for T cell activation to occur. In fact, without the second type of signals, the first type of signals can result in T cell anergy. The second type of signals, called co-stimulatory signals, is neither antigen-specific nor MHC-restricted, and can lead to a full proliferation response of T cells and induction of T cell effector functions in the presence of the first type of signals. As used herein, "dendritic cell" is to include, but not be limited to a pulsed dendritic cell, a foster cell or a dendritic cell hybrid.

The term "immune effector cells" refers to cells capable of binding an antigen or which mediate an immune response. These cells include, but are not limited to, T cells, B cells, monocytes, macrophages, NK cells and cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates. Certain diseased tissue expresses specific antigens and CTLs specific for these antigens have been identified. For example, approximately 80% of melanomas express the antigen known as gp100.

A "naïve" cell is a cell that has never been exposed to an antigen.

The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (morphologically, genetically, or phenotypically) to the parent cell. By "expanded" is meant any proliferation or division of cells.

A "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

The terms "major histocompatibility complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC complex is also known as the HLA complex. The proteins encoded by the MHC complex are known as "MHC molecules" and are classified into class I and class II MHC molecules. Class I MHC molecules include membrane heterodimeric proteins made up of an α chain encoded in the MHC associated noncovalently with β 2-microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to CD8+ T cells. Class I molecules include HLA-A, -B, and -C in humans. Class II MHC molecules also include membrane heterodimeric proteins consisting of noncovalently associated α and β chains. Class II MHC are known to participate in antigen presentation to CD4+ T cells and, in humans, include HLA-DP, -DQ, and DR. The term "MHC restriction" refers to a characteristic of T cells that permits them to recognize antigen only after it is processed and the resulting antigenic peptides are displayed in association with either a self class I or class II MHC molecule. Methods of identifying and comparing MHC are well known in the art and are described in Allen, M. et al. (1994) *Human Imm.* 40:25-32; Santamaria, P. et al. (1993) *Human Imm.* 37:39-50 and Hurley, C.K. et al. (1997) *Tissue Antigens* 50:401-415.

The term "peptide" is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunit may be linked by other bonds, e.g. ester, ether, etc. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an

oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative". For example, where the purpose of the experiment is to determine a correlation of an altered expression level of a gene with a particular type of cancer, it is generally preferable to use a positive control (a subject or a sample from a subject, carrying such alteration and exhibiting syndromes characteristic of that disease), and a negative control (a subject or a sample from a subject lacking the altered expression and clinical syndrome of that disease).

"PCR primers" refer to primers used in "polymerase chain reaction" or "PCR," a method for amplifying a DNA base sequence using a heat-stable polymerase such as Taq polymerase, and two oligonucleotide primers, one complementary to the (+)-strand at one end of the sequence to be amplified and the other complementary to the (-)-strand at the other end. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation produce exponential and highly specific amplification of the desired sequence. (See, e.g., PCR 2: A PRACTICAL APPROACH, *Supra*). PCR also can be used to detect the existence of the defined sequence in a DNA sample.

"Host cell" or "recipient cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of exogenous nucleic acid molecules, polynucleotides and/or proteins. It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. The cells may be procaryotic or eucaryotic, and include but are not limited to bacterial cells, yeast cells, animal cells, and mammalian cells, e.g., murine, rat, simian or human. An "antibody" is an immunoglobulin molecule capable of binding an antigen. As used herein, the term encompasses not only intact immunoglobulin molecules, but also anti-idiotypic antibodies, mutants, fragments, fusion proteins,

humanized proteins and modifications of the immunoglobulin molecule that comprise an antigen recognition site of the required specificity.

An "antibody complex" is the combination of antibody (as defined above) and its binding partner or ligand.

5 A native antigen is a polypeptide, protein or a fragment containing an epitope, which induces an immune response in the subject.

The term "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, does not require "isolation" to distinguish it from its naturally occurring counterpart. In addition, a "concentrated", "separated" or "diluted" polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, which differs from the naturally occurring counterpart in its primary sequence or for example, by its glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally occurring counterpart by its primary sequence, or alternatively, by another characteristic such as glycosylation pattern. Although not explicitly stated for each of the inventions disclosed herein, it is to be understood that all of the above embodiments for each of the compositions disclosed below and under the appropriate conditions, are provided by this invention. Thus, a non-naturally occurring polynucleotide is provided as a separate embodiment from the isolated naturally occurring polynucleotide. A protein produced in a bacterial cell is provided as a separate embodiment from the naturally occurring protein isolated from a eucaryotic cell in which it is produced in nature.

30 An "isolated" or "enriched" population of cells is "substantially free" of cells and materials with which it is associated in nature. By "substantially free" or

"substantially pure", means at least 50% of the population are the desired cell type, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90%.

5 A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent, solid support or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

10 As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

15 An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

20 This invention provides novel, synthetic peptide sequences which are useful as components of melanoma vaccines and to expand immune effector cells that are specific for melanoma. The peptides, FLDQVAFXV (Seq. ID No. 1) and FLFSWYAXV (Seq. ID No. 3), wherein X represents any amino acid, are particularly useful in that they have been shown to sensitize Hurley R1000 tumor
25 infiltrating lymphocytes and interferon- γ release (cytotoxic T cell specific for the G9-209 epitope encoded by the human melanoma associate protein gp100). These assay methods are known in the art and disclosed in Zugel, U. et al. (1998) *J. Immunol.* 161:1705-1709 and Salgaller, M.L. et al. (1995) *Infra*. The peptide sequences of the present invention differ from the natural epitope (ITDQVPFVSV)
30 (Seq. ID No. 5) in two ways: (1) they contain mutation sin the putative HLA-A2 binding domain (amino acid residues 1, 2, and 9) conferring tighter binding to the

MHC, and (2) they contain mutations in the putative T cell receptor-binding domain (amino acid residues 3-8) resulting in an apparent increased avidity for the T cell receptor. They also are novel over the synthetic peptides disclosed by Salgaller, et al. (1995) *Cancer Res.* 55:4972-4979.

This invention provides polynucleotides encoding polypeptides comprising the sequences FLDQVAFXV (Seq. ID No. 1) or FLFSWYAXV (Seq. ID No. 3), wherein X is any amino acid, and the complements of these polynucleotides. As used herein, the term "polynucleotide" encompasses DNA, RNA and nucleic acid mimetics. In addition to the sequences shown in Seq. ID Nos. 2 and 4, or their complements, this invention also provides the anti-sense polynucleotide stand, e.g. antisense RNA to these sequences or their complements. One can obtain an antisense RNA using the sequences provided in Seq. ID Nos. 2 and 4, and the methodology described in Vander Krol, et al. (1988) *BioTechniques* 6:958.

The polynucleotides can be conjugated to a detectable marker, e.g., an enzymatic label or a radioisotope for detection of nucleic acid and/or expression of the gene in a cell. A wide variety of appropriate detectable markers are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples. Briefly, this invention further provides a method for detecting a single-stranded or its complement, by contacting target single-stranded polynucleotides with a labeled, single-stranded polynucleotide (a probe) which is at least 4, and more preferably at least 5 or 6 and most preferably at least 10 of the 10 nucleotides shown in Seq. ID NO. 2 or 4, (or the corresponding complement) under conditions permitting hybridization (preferably moderately stringent hybridization conditions) of

complementary single-stranded polynucleotides, or more preferably, under highly stringent hybridization conditions. Hybridized polynucleotide pairs are separated from un-hybridized, single-stranded polynucleotides. The hybridized polynucleotide pairs are detected using methods well known to those of skill in the art and set forth, for example, in Sambrook, et al. (1989) *Supra*.

The polynucleotides of this invention can be replicated using PCR. PCR technology is the subject matter of United States Patent Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202 and described in PCR: THE POLYMERASE CHAIN REACTION (Mullis et al. eds, Birkhauser Press, Boston (1994)) and references cited therein.

Alternatively, one of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to replicate the DNA. Accordingly, this invention also provides a process for obtaining the polynucleotides of this invention by providing the linear sequence of the polynucleotide, appropriate primer molecules, chemicals such as enzymes and instructions for their replication and chemically replicating or linking the nucleotides in the proper orientation to obtain the polynucleotides. In a separate embodiment, these polynucleotides are further isolated. Still further, one of skill in the art can insert the polynucleotide into a suitable replication vector and insert the vector into a suitable host cell (prokaryotic or eucaryotic) for replication and amplification. The DNA so amplified can be isolated from the cell by methods well known to those of skill in the art. A process for obtaining polynucleotides by this method is further provided herein as well as the polynucleotides so obtained.

RNA can be obtained by first inserting a DNA polynucleotide into a suitable host cell. The DNA can be inserted by any appropriate method, e.g., by the use of an appropriate gene delivery vehicle (e.g., liposome, plasmid or vector) or by electroporation. When the cell replicates and the DNA is transcribed into RNA; the RNA can then be isolated using methods well known to those of skill in the art, for example, as set forth in Sambrook, et al. (1989) *Supra*. For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook, et al. (1989) *Supra* or extracted

by nucleic-acid-binding resins following the accompanying instructions provided by manufactures.

Polynucleotides having at least 4 contiguous nucleotides, and more preferably at least 5 or 6 contiguous nucleotides and most preferably at least 10 contiguous nucleotides, and exhibiting sequence complementarity or homology to Seq. ID Nos.2 or 4, find utility as hybridization probes.

It is known in the art that a "perfectly matched" probe is not needed for a specific hybridization. Minor changes in probe sequence achieved by substitution, deletion or insertion of a small number of bases do not affect the hybridization specificity. In general, as much as 20% base-pair mismatch (when optimally aligned) can be tolerated. Preferably, a probe useful for detecting the aforementioned mRNA is at least about 80% identical to the homologous region of comparable size contained in the previously identified sequences identified by Seq. ID Nos. 2 or 4, which correspond to previously characterized genes or Seq. ID Nos. 2. More preferably, the probe is 85% identical to the corresponding gene sequence after alignment of the homologous region; even more preferably, it exhibits 90% identity.

These probes can be used in radioassays (*e.g.* Southern and Northern blot analysis) to detect or monitor various cells or tissue containing these cells. The probes also can be attached to a solid support or an array such as a chip for use in high throughput screening assays for the detection of expression of the gene corresponding to one or more polynucleotide(s) of this invention. Accordingly, this invention also provides at least one probe as defined above of the transcripts identified as Seq. ID No. 2 or 4, or its complement, attached to a solid support for use in high throughput screens.

The polynucleotides of this invention also can be used for comparison to known and unknowns sequences using a computer-based method to match a sample sequence with known sequences. Thus, this invention also provides the polynucleotides of this invention in a computer database or in computer readable form, including applications utilizing the internet.

A linear search through such a database may be used. Alternatively, the polynucleotide sequence can be converted into a unique numeric representation. The comparison aspects may be implemented in hardware or software, or a combination of both. Preferably, these aspects of the invention are implemented in computer programs executing on a programable computer comprising a processor, a data storage system (including volatile and non-volatile memory and/or storage elements), at least one input device, and at least one output device. Data input through one or more input devices for temporary or permanent storage in the data storage system includes sequences, and may include previously generated polynucleotides and codes for known and/or unknown sequences. Program code is applied to the input data to perform the functions described above and generate output information. The output information is applied to one or more output devices, in known fashion.

Each such computer program is preferably stored on a storage media or device (e.g., ROM or magnetic diskette) readable by a general or special purpose programmable computer, for configuring and operating the computer when the storage media or device is read by the computer to perform the procedures described herein. The inventive system may also be considered to be implemented as a computer-readable storage medium, configured with a computer program, where the storage medium so configured causes a computer to operate in a specific and predefined manner to perform the functions described herein.

The polynucleotides of the present invention also can serve as primers for the detection of genes or gene transcripts that are expressed in APC, for example, to confirm transduction of the polynucleotides into host cells. In this context, amplification means any method employing a primer-dependent polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of *E.coli* DNA polymerase, and reverse transcriptase. A preferred length of the primer is the same as that identified for probes, above.

The invention further provides the isolated polynucleotide operatively linked to a promoter of RNA transcription, as well as other regulatory sequences for replication and/or transient or stable expression of the DNA or RNA. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct transcription of RNA off the DNA molecule. Examples of such promoters are SP6, T4 and T7. In certain embodiments, cell-specific promoters are used for cell-specific expression of the inserted polynucleotide. Vectors which contain a promoter or a promoter/enhancer, with termination codons and selectable marker sequences, as well as a cloning site into which an inserted piece of DNA can be operatively linked to that promoter are well known in the art and commercially available. For general methodology and cloning strategies, see GENE EXPRESSION TECHNOLOGY (Goeddel ed., Academic Press, Inc. (1991)) and references cited therein and VECTORS: ESSENTIAL DATA SERIES (Gacesa and Ramji, eds., John Wiley & Sons, N.Y. (1994)), which contains maps, functional properties, commercial suppliers and a reference to GenEMBL accession numbers for various suitable vectors. Preferable, these vectors are capable of transcribing RNA *in vitro* or *in vivo*.

Expression vectors containing these nucleic acids are useful to obtain host vector systems to produce proteins and polypeptides. It is implied that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include plasmids, viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, etc. Adenoviral vectors are particularly useful for introducing genes into tissues *in vivo* because of their high levels of expression and efficient transformation of cells both *in vitro* and *in vivo*. When a nucleic acid is inserted into a suitable host cell, e.g., a procaryotic or a eucaryotic cell and the host cell replicates, the protein can be recombinantly produced. Suitable host cells will depend on the vector and can include mammalian cells, animal cells, human cells, simian cells, insect cells, yeast cells, and bacterial cells constructed using well known methods. See Sambrook, et al. (1989) *Supra*. In addition to the use of viral vector for insertion of exogenous nucleic acid into cells, the nucleic

acid can be inserted into the host cell by methods well known in the art such as transformation for bacterial cells; transfection using calcium phosphate precipitation for mammalian cells; or DEAE-dextran; electroporation; or microinjection. See Sambrook et al. (1989) *Supra* for this methodology. Thus, this invention also provides a host cell, e.g. a mammalian cell, an animal cell (rat or mouse), a human cell, or a procaryotic cell such as a bacterial cell, containing a polynucleotide encoding a protein or polypeptide or antibody.

When the vectors are used for gene therapy *in vivo* or *ex vivo*, a pharmaceutically acceptable vector is preferred, such as a replication-incompetent retroviral or adenoviral vector. Pharmaceutically acceptable vectors containing the nucleic acids of this invention can be further modified for transient or stable expression of the inserted polynucleotide. As used herein, the term "pharmaceutically acceptable vector" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the nucleic acid into dividing cells. An example of such a vector is a "replication-incompetent" vector defined by its inability to produce viral proteins, precluding spread of the vector in the infected host cell. An example of a replication-incompetent retroviral vector is LNL6 (Miller, A.D. et al. (1989) *BioTechniques* 7:980-990). The methodology of using replication-incompetent retroviruses for retroviral-mediated gene transfer of gene markers is well established (Correll, et al. (1989) *PNAS USA* 86:8912; Bordignon (1989) *PNAS USA* 86:8912-52; Culver, K. (1991) *PNAS USA* 88:3155; and Rill, D.R. (1991) *Blood* 79(10):2694-700).

These host cells containing the polynucleotides of this invention are useful for the recombinant replication of the polynucleotides and for the recombinant production of peptides. Alternatively, the cells may be used to induce an immune response in a subject in the methods described herein. When the host cells are antigen presenting cells, they can be used to expand a population of immune effector cells such as tumor infiltrating lymphocytes which in turn are useful in adoptive immunotherapies.

Further provided herein are polypeptides comprising the sequences FLDQVAFXV (Seq. ID No. 1) and FLFSWYAXV (Seq. ID No. 3), wherein X is

any amino acid. Further provided are polypeptides comprising these sequences wherein the polypeptides are preferentially recognized by gp100 specific cytotoxic T lymphocytes.

5 The proteins and polypeptides of this invention can be obtained by chemical synthesis using a commercially available automated peptide synthesizer such as those manufactured by Perkin Elmer/Applied Biosystems, Inc., Model 430A or 431A, Foster City, CA, USA. The synthesized protein or polypeptide can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Accordingly, this invention also provides a process for
10 chemically synthesizing the proteins of this invention by providing the sequence of the protein and reagents, such as amino acids and enzymes and linking together the amino acids in the proper orientation and linear sequence.

Alternatively, the proteins and polypeptides can be obtained by well-known recombinant methods as described above using the host cell and vector
15 systems described above.

Also provided by this application are the polypeptides and proteins described herein conjugated to a detectable agent for use in the diagnostic methods. For example, detectably labeled proteins and polypeptides can be bound to a column and used for the detection and purification of antibodies. They also
20 are useful as immunogens for the production of antibodies as described below. The proteins and fragments of this invention are useful in an *in vitro* assay system to screen for agents or drugs, which modulate cellular processes.

The proteins of this invention also can be combined with various liquid phase carriers, such as sterile or aqueous solutions, pharmaceutically acceptable carriers, suspensions and emulsions. Examples of non-aqueous solvents include
25 propyl ethylene glycol, polyethylene glycol and vegetable oils. When used to prepare antibodies, the carriers also can include an adjuvant that is useful to non-specifically augment a specific immune response. A skilled artisan can easily determine whether an adjuvant is required and select one. However, for the
30 purpose of illustration only, suitable adjuvants include, but are not limited to Freund's Complete and Incomplete, mineral salts and polynucleotides.

Also provided by this invention is an antibody capable of specifically forming a complex with the polypeptides of this invention. The term "antibody" includes polyclonal antibodies and monoclonal antibodies. The antibodies include, but are not limited to mouse, rat, and rabbit or human antibodies. The antibodies are useful to identify and purify polypeptides and APCs expressing the polypeptides.

Laboratory methods for producing polyclonal antibodies and monoclonal antibodies, as well as deducing their corresponding nucleic acid sequences, are known in the art, see Harlow and Lane (1988) *Supra* and Sambrook, et al. (1989) *Supra*. The monoclonal antibodies of this invention can be biologically produced by introducing protein or a fragment thereof into an animal, e.g., a mouse or a rabbit. The antibody producing cells in the animal are isolated and fused with myeloma cells or heteromyeloma cells to produce hybrid cells or hybridomas. Accordingly, the hybridoma cells producing the monoclonal antibodies of this invention also are provided.

Thus, using the protein or fragment thereof, and well known methods, one of skill in the art can produce and screen the hybridoma cells and antibodies of this invention for antibodies having the ability to bind the proteins or polypeptides.

If a monoclonal antibody being tested binds with the protein or polypeptide, then the antibody being tested and the antibodies provided by the hybridomas of this invention are equivalent. It also is possible to determine without undue experimentation, whether an antibody has the same specificity as the monoclonal antibody of this invention by determining whether the antibody being tested prevents a monoclonal antibody of this invention from binding the protein or polypeptide with which the monoclonal antibody is normally reactive. If the antibody being tested competes with the monoclonal antibody of the invention as shown by a decrease in binding by the monoclonal antibody of this invention, then it is likely that the two antibodies bind to the same or a closely related epitope. Alternatively, one can pre-incubate the monoclonal antibody of this invention with a protein with which it is normally reactive, and determine if

the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of this invention.

5 The term "antibody" also is intended to include antibodies of all isotypes. Particular isotypes of a monoclonal antibody can be prepared either directly by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class switch variants using the procedure described
10 in Stepleski, et al. (1985) *Proc. Natl. Acad. Sci.* **82**:8653 or Spira, et al. (1984) *J. Immunol. Methods* **74**:307.

This invention also provides biological active fragments of the polyclonal and monoclonal antibodies described above. These "antibody fragments" retain some ability to selectively bind with its antigen or immunogen. Such antibody
15 fragments can include, but are not limited to:

- (1) Fab,
- (2) Fab',
- (3) F(ab')₂,
- (4) Fv, and
- 20 (5) SCA

A specific example of "a biologically active antibody fragment" is a CDR region of the antibody. Methods of making these fragments are known in the art, see for example, Harlow and Lane (1988) *Supra*.

25 The antibodies of this invention also can be modified to create chimeric antibodies and humanized antibodies (Oi, et al. (1986) *BioTechniques* **4**(3):214). Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species.

The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the invention can also be
30 accomplished by one of ordinary skill in the art by producing anti-idiotypic antibodies (Herlyn, et al. (1986) *Science* **232**:100). An anti-idiotypic antibody is

an antibody which recognizes unique determinants present on the monoclonal antibody produced by the hybridoma of interest.

Idiotypic identity between monoclonal antibodies of two hybridomas demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using antibodies to the epitopic determinants on a monoclonal antibody it is possible to identify other hybridomas expressing monoclonal antibodies of the same epitopic specificity.

It is also possible to use the anti-idiotypic technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the mirror image of the epitope bound by the first monoclonal antibody. Thus, in this instance, the anti-idiotypic monoclonal antibody could be used for immunization for production of these antibodies.

As used in this invention, the term "epitope" is meant to include any determinant having specific affinity for the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The antibodies of this invention can be linked to a detectable agent or label. There are many different labels and methods of labeling known to those of ordinary skill in the art.

The coupling of antibodies to low molecular weight haptens can increase the sensitivity of the assay. The haptens can then be specifically detected by means of a second reaction. For example, it is common to use haptens such as biotin, which reacts avidin, or dinitrophenyl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies. See Harlow and Lane (1988) *Supra*.

The monoclonal antibodies of the invention also can be bound to many different carriers. Thus, this invention also provides compositions containing the

antibodies and another substance, active or inert. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

Compositions containing the antibodies, fragments thereof or cell lines which produce the antibodies, are encompassed by this invention. When these compositions are to be used pharmaceutically, they are combined with a pharmaceutically acceptable carrier.

The polypeptides of this invention also can be pulsed into antigen presenting cells using the methods described herein. Antigen-presenting cells, include, but are not limited to dendritic cells (DCs), monocytes/macrophages, B lymphocytes or other cell type(s) expressing the necessary MHC/co-stimulatory molecules. The methods described below focus primarily on DCs which are the most potent, preferred APCs. These host cells containing the polypeptides or proteins are further provided.

Isolated host cells which present the polypeptides of this invention in the context of MHC molecules are further useful to expand and isolate a population of educated, antigen-specific immune effector cells. The immune effector cells, e.g., cytotoxic T lymphocytes, are produced by culturing naïve immune effector cells with antigen-presenting cells which present the polypeptides in the context of MHC molecules on the surface of the APCs. The population can be purified using methods known in the art, e.g., FACS analysis or ficoll gradient. The methods to generate and culture the immune effector cells as well as the populations produced thereby also are the inventor's contribution and invention. Pharmaceutical compositions comprising the cells and pharmaceutically acceptable carriers are useful in adoptive immunotherapy. Prior to administration *in vivo*, the immune effector cells are screened *in vitro* for their ability to lyse melanoma tumor cells.

In one embodiment, the immune effector cells and/or the APCs are genetically modified. Using standard gene transfer, genes coding for co-stimulatory molecules and/or stimulatory cytokines can be inserted prior to, concurrent to or subsequent to expansion of the immune effector cells.

5 This invention also provides methods of inducing an immune response in a subject, comprising administering to the subject an effective amount of the polypeptides described above under the conditions that induce an immune response to the polypeptide. The polypeptides can be administered in formulations or as polynucleotides encoding the polypeptides. The
10 polynucleotides can be administered in a gene delivery vehicle or by inserting into a host cell which in turn recombinantly transcribes, translates and processed the encoded polypeptide. Isolated host cells containing the polynucleotides of this invention in a pharmaceutically acceptable carrier can therefore combined with appropriate and effective amount of an adjuvant, cytokine or co-stimulatory
15 molecule for an effective vaccine regimen. In one embodiment, the host cell is an APC such as a dendritic cell. The host cell can be further modified by inserting of a polynucleotide coding for an effective amount of either or both of a cytokine a co-stimulatory molecule.

The methods of this invention can be further modified by co-administering
20 an effective amount of a cytokine or co-stimulatory molecule to the subject.

This invention also provides compositions containing any of the above-mentioned proteins, polypeptides, polynucleotides, vectors, cells, antibodies and fragments thereof, and an acceptable solid or liquid carrier. When the compositions are used pharmaceutically, they are combined with a
25 "pharmaceutically acceptable carrier" for diagnostic and therapeutic use. These compositions also can be used for the preparation of medicaments for the diagnosis and treatment of diseases such as melanoma.

The following materials and methods are intended to illustrate, but not
30 limit this invention and to illustrate how to make and use the inventions described above.

Materials and Methods

Production of the Polypeptides of the Invention

Most preferably, isolated peptides of the present invention can be synthesized using an appropriate solid state synthetic procedure. Steward and Young, Solid Phase Peptide Synthesis, Freemantle, San Francisco, Calif. (1968). A preferred method is the Merrifield process. Merrifield, (1967) *Recent Progress in Hormone Res.* 23:451. The antigenic activity of these peptides may conveniently be tested using, for example, the assays as described herein.

Once an isolated peptide of the invention is obtained, it may be purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. For immunoaffinity chromatography, an epitope may be isolated by binding it to an affinity column comprising antibodies that were raised against that peptide, or a related peptide of the invention, and were affixed to a stationary support.

Alternatively, affinity tags such as hexa-His (Invitrogen), Maltose binding domain (New England Biolabs), influenza coat sequence (Kolodziej, et al. (1991) *Methods Enzymol.* 194:508-509), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. Isolated peptides can also be physically characterized using such techniques as proteolysis, nuclear magnetic resonance, and x-ray crystallography.

Also included within the scope of the invention are antigenic peptides that are differentially modified during or after translation, e.g., by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand, (Ferguson, et al. (1988) *Ann. Rev. Biochem.* 57:285-320).

Isolation, Culturing and Expansion of APCs, Including Dendritic Cells

The following is a brief description of two fundamental approaches for the isolation of APC. These approaches involve (1) isolating bone marrow precursor cells (CD34⁺) from blood and stimulating them to differentiate into APC; or (2) collecting the precommitted APCs from peripheral blood. In the first approach, the patient must be treated with cytokines such as GM-CSF to boost the number of circulating CD34⁺ stem cells in the peripheral blood.

The second approach for isolating APCs is to collect the relatively large numbers of precommitted APCs already circulating in the blood. Previous techniques for isolating committed APCs from human peripheral blood have involved combinations of physical procedures such as metrizamide gradients and adherence/nonadherence steps (Freudenthal, P.S. et al. (1990) *PNAS* 87:7698-7702); Percoll gradient separations (Mehta-Damani, et al. (1994) *J. Immunol.* 153:996-1003); and fluorescence activated cell sorting techniques (Thomas, R. et al. (1993) *J. Immunol.* 151:6840-52).

One technique for separating large numbers of cells from one another is known as countercurrent centrifugal elutriation (CCE). In this technique, cells are subject to simultaneous centrifugation and a washout stream of buffer which is constantly increasing in flow rate. The constantly increasing countercurrent flow of buffer leads to fractional cell separations that are largely based on cell size.

In one aspect of the invention, the APC are precommitted or mature dendritic cells which can be isolated from the white blood cell fraction of a mammal, such as a murine, simian or a human (See, e.g., WO 96/23060). The white blood cell fraction can be from the peripheral blood of the mammal. This method includes the following steps: (a) providing a white blood cell fraction obtained from a mammalian source by methods known in the art such as leukophoresis; (b) separating the white blood cell fraction of step (a) into four or more subfractions by countercurrent centrifugal elutriation, (c) stimulating conversion of monocytes in one or more fractions from step (b) to dendritic cells by contacting the cells with calcium ionophore, GM-CSF and IL-13 or GM-CSF and IL-4, (d) identifying the dendritic cell-enriched fraction from step (c), and (e)

collecting the enriched fraction of step (d), preferably at about 4°C. One way to identify the dendritic cell-enriched fraction is by fluorescence-activated cell sorting. The white blood cell fraction can be treated with calcium ionophore in the presence of other cytokines, such as recombinant (rh) rhIL-12, rhGM-CSF, or rhIL-4. The cells of the white blood cell fraction can be washed in buffer and suspended in $\text{Ca}^{++}/\text{Mg}^{++}$ free media prior to the separating step. The white blood cell fraction can be obtained by leukapheresis. The dendritic cells can be identified by the presence of at least one of the following markers: HLA-DR, HLA-DQ, or B7. 2, and the simultaneous absence of the following markers: CD3, CD14, CD16, 56, 57, and CD 19, 20. Monoclonal antibodies specific to these cell surface markers are commercially available.

More specifically, the method requires collecting an enriched collection of white cells and platelets from leukapheresis that is then further fractionated by countercurrent centrifugal elutriation (CCE) (Abrahamsen, T.G. et al. (1991) *J. Clin. Apheresis*. 6:48-53). Cell samples are placed in a special elutriation rotor. The rotor is then spun at a constant speed of, for example, 3000 rpm. Once the rotor has reached the desired speed, pressurized air is used to control the flow rate of cells. Cells in the elutriator are subjected to simultaneous centrifugation and a washout stream of buffer which is constantly increasing in flow rate. This results in fractional cell separations based largely but not exclusively on differences in cell size.

Quality control of APC and more specifically DC collection and confirmation of their successful activation in culture is dependent upon a simultaneous multi-color FACS analysis technique which monitors both monocytes and the dendritic cell subpopulation as well as possible contaminant T lymphocytes. It is based upon the fact that DCs do not express the following markers: CD3 (T cell); CD14 (monocyte); CD16, 56, 57 (NK/LAK cells); CD19, 20 (B cells). At the same time, DCs do express large quantities of HLA-DR, significant HLA-DQ and B7.2 (but little or no B7.1) at the time they are circulating in the blood (in addition they express Leu M7 and M9, myeloid markers which are also expressed by monocytes and neutrophils).

When combined with a third color reagent for analysis of dead cells, propidium iodide (PI), it is possible to make positive identification of all cell subpopulations (see Table 1):

TABLE 1
FACS analysis of fresh peripheral cell subpopulations

	Color #1	Color #2	Color #3
	Cocktail	HLA-DR	PI
	3/14/16/19/20/56/57		
Live Dendritic cells	Negative	Positive	Negative
Live Monocytes	Positive	Positive	Negative
Live Neutrophils	Negative	Negative	Negative
Dead Cells	Variable	Variable	Positive

5 Additional markers can be substituted for additional analysis:

Color #1: CD3 alone, CD14 alone, etc.; Leu M7 or Leu M9; anti-Class I, etc.

Color #2: HLA-Dq, B7.1, B7.2, CD25 (IL2r), ICAM, LFA-3, etc.

10 The goal of FACS analysis at the time of collection is to confirm that the DCs are enriched in the expected fractions, to monitor neutrophil contamination, and to make sure that appropriate markers are expressed. This rapid bulk collection of enriched DCs from human peripheral blood, suitable for clinical applications, is absolutely dependent on the analytic FACS technique described
15 above for quality control. If need be, mature DCs can be immediately separated from monocytes at this point by fluorescent sorting for "cocktail negative" cells. It may not be necessary to routinely separate DCs from monocytes because, as will be detailed below, the monocytes themselves are still capable of differentiating into DCs or functional DC-like cells in culture.

20 Once collected, the DC rich/monocyte APC fractions (usually 150 through 190) can be pooled and cryopreserved for future use, or immediately placed in short term culture.

Alternatively, others have reported that a method for upregulating (activating) dendritic cells and converting monocytes to an activated dendritic cell phenotype. This method involves the addition of calcium ionophore to the culture media convert monocytes into activated dendritic cells. Adding the calcium ionophore A23187, for example, at the beginning of a 24-48 hour culture period resulted in uniform activation and dendritic cell phenotypic conversion of the pooled "monocyte plus DC" fractions: characteristically, the activated population becomes uniformly CD14 (Leu M3) negative, and upregulates HLA-DR, HLA-DQ, ICAM-1, B7.1, and B7.2. Furthermore this activated bulk population functions as well on a small numbers basis as a further purified.

Specific combination(s) of cytokines have been used successfully to amplify (or partially substitute) for the activation/conversion achieved with calcium ionophore: these cytokines include but are not limited to purified or recombinant ("rh") rhGM-CSF, rhIL-2, and rhIL-4. Each cytokine when given alone is inadequate for optimal upregulation.

Presentation of Antigen to the APC

For purposes of immunization, the antigenic peptides (Seq. ID No. 1 and No. 3) can be delivered to antigen-presenting cells as protein/peptide or in the form of cDNA encoding the protein/peptide. Antigen-presenting cells (APCs) can consist of dendritic cells (DCs), monocytes/macrophages, B lymphocytes or other cell type(s) expressing the necessary MHC/co-stimulatory molecules. The methods described below focus primarily on DCs which are the most potent, preferred APCs.

Pulsing is accomplished *in vitro/ex vivo* by exposing APCs to the antigenic protein or peptide(s) of this invention. The protein or peptide(s) are added to APCs at a concentration of 1-10 μ m for approximately 3 hours. Pulsed APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

Protein/peptide antigen can also be delivered *in vivo* with adjuvant via the intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

Paglia, et al. (1996) *J. Exp. Med.* **183**:317-322 has shown that APC incubated with whole protein *in vitro* were recognized by MHC class I-restricted CTLs, and that immunization of animals with these APCs led to the development of antigen-specific CTLs *in vivo*. In addition, several different techniques have been described which lead to the expression of antigen in the cytosol of APCs, such as DCs. These include (1) the introduction into the APCs of RNA isolated from tumor cells, (2) infection of APCs with recombinant vectors to induce endogenous expression of antigen, and (3) introduction of tumor antigen into the DC cytosol using liposomes. (See Boczkowski, D. et al. (1996) *J. Exp. Med.* **184**:465-472; Rouse, et al. (1994) *J. Virol.* **68**:5685-5689; and Nair, et al. (1992) *J. Exp. Med.* **175**:609-612).

Foster Antigen Presenting Cells

Foster antigen presenting cells are particularly useful as target. Foster APCs are derived from the human cell line 174xCEM.T2, referred to as T2, which contains a mutation in its antigen processing pathway that restricts the association of endogenous peptides with cell surface MHC class I molecules (Zweerink, et al. (1993) *J. Immunol.* **150**:1763-1771). This is due to a large homozygous deletion in the MHC class II region encompassing the genes TAP1, TAP2, LMP1, and LMP2, which are required for antigen presentation to MHC class I-restricted CD8⁺ CTLs. In effect, only "empty" MHC class I molecules are presented on the surface of these cells. Exogenous peptide added to the culture medium binds to these MHC molecules provided that the peptide contains the allele-specific binding motif. These T2 cells are referred to herein as "foster" APCs. They can be used in conjunction with this invention to present antigen(s).

Transduction of T2 cells with specific recombinant MHC alleles allows for redirection of the MHC restriction profile. Libraries tailored to the recombinant

allele will be preferentially presented by them because the anchor residues will prevent efficient binding to the endogenous allele.

High level expression of MHC molecules makes the APC more visible to the CTLs. Expressing the MHC allele of interest in T2 cells using a powerful transcriptional promoter (e.g., the CMV promoter) results in a more reactive APC (most likely due to a higher concentration of reactive MHC-peptide complexes on the cell surface).

Expansion of Immune Effector Cells

The present invention makes use of these APCs to stimulate production of an enriched population of antigen-specific immune effector cells. The antigen-specific immune effector cells are expanded at the expense of the APCs, which die in the culture. The process by which naïve immune effector cells become educated by other cells is described essentially in Coulie (1997) *Molec. Med Today* 3:261-268.

The APCs prepared as described above are mixed with naïve immune effector cells. Preferably, the cells may be cultured in the presence of a cytokine, for example IL2. Because dendritic cells secrete potent immunostimulatory cytokines, such as IL12, it may not be necessary to add supplemental cytokines during the first and successive rounds of expansion. In any event, the culture conditions are such that the antigen-specific immune effector cells expand (*i.e.* proliferate) at a much higher rate than the APCs. Multiple infusions of APCs and optional cytokines can be performed to further expand the population of antigen-specific cells.

In one embodiment, the immune effector cells are T cells. In a separate embodiment, the immune effector cells can be genetically modified by transduction with a transgene coding for example, IL-2, IL-11 or IL-13. Methods for introducing transgenes *in vitro*, *ex vivo* and *in vivo* are well known in the art. See Sambrook, et al. (1989) *Supra*.

Vectors Useful in Genetic Modifications

In general, genetic modifications of cells employed in the present invention are accomplished by introducing a vector containing a polypeptide or transgene encoding a heterologous or an altered antigen. A variety of different gene transfer vectors, including viral as well as non-viral systems can be used. Viral vectors useful in the genetic modifications of this invention include, but are not limited to adenovirus, adeno-associated virus vectors, retroviral vectors and adeno-retroviral chimeric vectors. APC and immune effector cells can be modified using the methods described below or by any other appropriate method known in the art.

Construction of Recombinant Adenoviral Vectors or Adeno-Associated Virus Vectors

Adenovirus and adeno-associated virus vectors useful in the genetic modifications of this invention may be produced according to methods already taught in the art. (see, e.g., Karlsson, et al. (1986) *EMBO* 5:2377; Carter (1992) *Current Opinion in Biotechnology* 3:533-539; Muzyczka (1992) *Current Top. Microbiol. Immunol.* 158:97-129; GENE TARGETING: A PRACTICAL APPROACH (1992) ed. A. L. Joyner, Oxford University Press, NY). Several different approaches are feasible. Preferred is the helper-independent replication deficient human adenovirus system.

The recombinant adenoviral vectors based on the human adenovirus 5 (*Virology* 163:614-617, 1988) are missing essential early genes from the adenoviral genome (usually E1A/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products *in trans*. In place of the missing adenoviral genomic sequences, a transgene of interest can be cloned and expressed in cells infected with the replication deficient adenovirus. Although adenovirus-based gene transfer does not result in integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), and therefore is not stable, adenoviral vectors can be propagated in high titer and

transfect non-replicating cells. Human 293 cells, which are human embryonic kidney cells transformed with adenovirus E1A/E1B genes, typify useful permissive cell lines. However, other cell lines which allow replication-deficient adenoviral vectors to propagate therein can be used, including HeLa cells.

5 Additional references describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz, M.S., *Adenoviridae and Their Replication*, in Fields, B., et al. (eds.) *VIROLOGY*, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham, F. et al., pp. 109-128 in *METHODS IN MOLECULAR BIOLOGY*, Vol. 7: GENE
10 TRANSFER AND EXPRESSION PROTOCOLS, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, N., et al. (1995) *FASEB Journal* 9:190-199 Schreier, H (1994) *Pharmaceutica Acta Helvetiae* 68:145-159; Schneider and French (1993) *Circulation* 88:1937-1942; Curiel D.T., et al.(1992) *Human Gene Therapy* 3:147-154; Graham, F.L., et al., WO 95/00655 (5 January 1995); Falck-Pedersen, E.S.
15 WO 95/16772 (22 June 1995); Denefle, P. et al. WO 95/23867 (8 September 1995); Haddada, H. et al. WO 94/26914 (24 November 1994); Perricaudet, M. et al. WO 95/02697 (26 January 1995); Zhang, W. et al. WO 95/25071 (12 October 1995). A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix
20 Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996). See also, the papers by Vile, et al. (1997) *Nature Biotechnology* 15:840-841; Feng, et al.(1997) *Nature Biotechnology*, 15:866-870, describing the construction and use of adeno-retroviral chimeric vectors that can be employed for genetic
25 modifications.

Additional references describing AAV vectors which could be used in the methods of the present invention include the following: Carter, B., *HANDBOOK OF PARVOVIRUSES*, Vol. I, pp. 169-228, 1990; Berns, *VIROLOGY*, pp. 1743-1764 (Raven Press 1990); Carter, B. (1992) *Curr. Opin. Biotechnol.* 3:533-539; Muzyczka, N. (1992) *Current Topics in Micro and Immunol.* 158:92-129; Flotte,
30 T.R. et al. (1992) *Am. J. Respir. Cell Mol. Biol.* 7:349-356; Chatterjee, et al. (1995) *Ann. NY Acad. Sci.* 770:79-90; Flotte, T.R. et al. WO 95/13365 (18 May

1995); Trempe, J.P. et al., WO 95/13392 (18 May 1995); Kotin, R.(1994) *Human Gene Therapy* 5:793-801; Flotte, T.R. et al. (1995) *Gene Therapy* 2:357-362; Allen, J.M., WO 96/17947 (13 June 1996); and Du, et al. (1996) *Gene Therapy* 3:254-261.

5 APCs can be transduced with viral vectors encoding a relevant polypeptides. The most common viral vectors include recombinant poxviruses such as vaccinia and fowlpox virus (Bronte, et al. (1997) *PNAS* 94:3183-3188; Kim, et al. (1997) *J. Immunother.* 20:276-286) and, preferentially, adenovirus (Arthur, et al. (1997) *J. Immunol.* 159:1393-1403; Wan, et al. (1997) *Human Gene Therapy* 8:1355-1363; Huang, et al. (1995) *J. Virol.* 69:2257-2263). Retrovirus also may be used for transduction of human APCs (Marin, et al. (1996) *J. Virol.* 70:2957-2962).

10 *In vitro/ex vivo*, exposure of human DCs to adenovirus (Ad) vector at a multiplicity of infection (MOI) of 500 for 16-24 h in a minimal volume of serum-free medium reliably gives rise to transgene expression in 90-100% of DCs. The efficiency of transduction of DCs or other APCs can be assessed by immunofluorescence using fluorescent antibodies specific for the tumor antigen being expressed (Kim, et al. (1997) *J. Immunother.* 20:276-286). Alternatively, the antibodies can be conjugated to an enzyme (e.g. HRP) giving rise to a colored product upon reaction with the substrate. The actual amount of antigenic polypeptides being expressed by the APCs can be evaluated by ELISA.

15 Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

20 *In vivo* transduction of DCs, or other APCs, can be accomplished by administration of Ad (or other viral vectors) via different routes including intravenous, intramuscular, intranasal, intraperitoneal or cutaneous delivery. The preferred method is cutaneous delivery of Ad vector at multiple sites using a total dose of approximately 1×10^{10} - 1×10^{12} i.u. Levels of *in vivo* transduction can be roughly assessed by co-staining with antibodies directed against APC marker(s) and the TAA being expressed. The staining procedure can be carried out on

biopsy samples from the site of administration or on cells from draining lymph nodes or other organs where APCs (in particular DCs) may have migrated (Condon, et al. (1996) *Nature Med.* 2:1122-1128; Wan, et al. (1997) *Human Gene Therapy* 8:1355-1363). The amount of antigen being expressed at the site of injection or in other organs where transduced APCs may have migrated can be evaluated by ELISA on tissue homogenates.

Although viral gene delivery is more efficient, DCs can also be transduced *in vitro/ex vivo* by non-viral gene delivery methods such as electroporation, calcium phosphate precipitation or cationic lipid/plasmid DNA complexes (Arthur, et al. (1997) *Cancer Gene Therapy* 4:17-25). Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

In vivo transduction of DCs, or other APCs, can potentially be accomplished by administration of cationic lipid/plasmid DNA complexes delivered via the intravenous, intramuscular, intranasal, intraperitoneal or cutaneous route of administration. Gene gun delivery or injection of naked plasmid DNA into the skin also leads to transduction of DCs (Condon, et al. (1996) *Nature Med.* 2:1122-1128; Raz, et al (1994) *PNAS* 91:9519-9523). Intramuscular delivery of plasmid DNA may also be used for immunization (Rosato, et al. (1997) *Human Gene Therapy* 8:1451-1458).

The transduction efficiency and levels of transgene expression can be assessed as described above for viral vectors.

Adoptive Immunotherapy and Vaccines

The expanded populations of antigen-specific immune effector cells of the present invention also find use in adoptive immunotherapy regimes and as vaccines.

Adoptive immunotherapy methods involve, in one aspect, administering to a subject a substantially pure population of educated, antigen-specific immune effector cells made by culturing naïve immune effector cells with APCs as described above. Preferably, the APCs are dendritic cells.

In one embodiment, the adoptive immunotherapy methods described herein are autologous. In this case, the APCs are made using parental cells isolated from a single subject. The expanded population also employs T cells isolated from that subject. Finally, the expanded population of antigen-specific cells is administered to the same patient.

In a further embodiment, APCs or immune effector cells are administered with an effective amount of a stimulatory cytokine, such as IL-2 or a co-stimulatory molecule.

The agents identified herein as effective for their intended purpose can be administered to subjects having melanoma or to individuals susceptible to or at risk of developing melanoma. When the agent is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject. To determine patients that can be beneficially treated, a tumor regression can be assayed. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the efficacy and toxicity of the therapy.

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

The agents and compositions of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

More particularly, an agent of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route

including nasal, topical (including transdermal, aerosol, buccal and sublingual), parental (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease being treated.

Examples

Based on the idea that identification of new and better tumor antigens will lead to the design of superior vaccines, an empirical screening method known as SHPERE has been developed for the identification and optimization of MHC Class I-restricted CTL epitopes. The SPHERE approach utilizes combinatorial peptide libraries synthesized on polystyrene beads where each bead contains a pure population of a unique peptide that can be chemically released from the beads in discrete aliquots. Released peptide from pooled bead arrays are screened using a modified ^{51}Cr -release assay to identify peptide pools capable of activating a T cell of interest. By utilizing an iterative peptide pool/releasing strategy it is possible to screen more than 10^7 peptides in just a few days. Analysis of residual peptide on the corresponding positive beads (>100 pmoles) allows rapid and unambiguous identification of the epitope sequence. The inventor utilized this method by screening 5 million peptides (from a library of about 47 million peptides) for epitopes that react with an HLA-A-A2-restricted CD8^+ T cell with known specificity for the 209-217 peptide of human gp100. The synthetic 9-mer library was designed with a fixed high-affinity HLA-A-A2 epitope and a variable TCR epitope repertoire (sequence F-L-X-X-X-X-X-V, wherein X is any one of 19 amino acids). The library de-convolution strategy involved 3 iterations starting with pools of 10,000 peptides for the primary screen. In this manner, peptide sequences that are preferentially recognized by the gp100 209 peptide-specific CTL were identified and can be used for vaccines having the unique property of being able to reverse peripheral tolerance.

C57BL/6 mice can be used as an appropriate animal model to assay the use of the above compositions and methods in combination with other known or

yet undiscovered anti-tumor therapies. Animals are immunized against the polypeptides with an intravenous injection of 5×10^5 bone marrow-derived dendritic cells (DCs) transfected with adenovirus vector encoding the antigenic peptides of this invention. Uninfected DCs can served as a negative control. Two
5 weeks after immunization, the mice are challenged with a subcutaneous injection of 2×10^4 gp100 melanoma cells and tumor growth is monitored over time.

Dendritic cells derived from peripheral blood of a subject such as a human patient are transduced with adenovirus vector containing the polynucleotides of this invention at a multiplicity of infection of 200-500 for use in adoptive
10 immunotherapy. Approximately 24 hours after infection, the transfected dendritic cells ($1-5 \times 10^7$ cells) are administered to the patient IV or subcutaneously. The process is repeated 3-4 weeks later with up to 5 administrations of dendritic cells. Since it is possible to freeze dendritic cells and administer thawed cells, the subject does not have to be leukophoresed each time.

It is to be understood that while the invention has been described in
15 conjunction with the above embodiments, that the foregoing description and the following examples are intended to illustrate and not limit the scope of the invention. For example, any of the above-noted compositions and/or methods can be combined with known therapies or compositions. Other aspects, advantages
20 and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.